Antibodies against preselected peptides to map functional sites on the acetylcholine receptor

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Rabbit immune sera and mouse monoclonal antibodies were raised against the synthetic peptide Tyr-Cys-Glu-Ile-Ile-Val matching in sequence residues 127–132 of the α-subunit of all nicotinic acetylcholine receptors sequenced so far. Representative cholinergic ligands did not interfere with the binding of these antibodies to the receptor from *Torpedo marmorata*, indicating that this sequence is not part of the binding sites for cholinergic ligands. The applicability of antigenic sites analysis to the mapping of functional sites on receptor proteins is discussed.

Anti-peptide antibody *Monoclonal antibody* *Acetylcholine receptor* *Site mapping* *Antigenic site*

1. INTRODUCTION

Although the nicotinic acetylcholine receptor from electric tissue has been studied in more detail than any other neurotransmitter receptor [1], a full understanding of its structure-function relationship has not yet been achieved. With its genes cloned and its primary structure elucidated [2–8], however, the receptor is now accessible to new approaches in this direction. These include chemical modification of defined sequence positions, site-directed mutagenesis and, as we propose here, the application of antibodies prepared against short synthetic peptides matching in sequence predetermined sites on the receptor surface. Employing such antibodies, the sequence positions of their antigenic sites can be tested as to whether they overlap with functional sites of the receptor.

To obtain antibodies of the required specificity, the following conditions must be met: (i) The synthetic peptides must be sufficiently long to provide antigenic properties (4–6 amino acids) [9]. However, to avoid any ambiguity in antigenic site definition, this limiting size should not be exceeded. (ii) The obtained antibodies must specifically recognize the peptide region of the peptide-carrier conjugate employed in the immunisation procedure. (iii) The antibodies must recognize the receptor both in its natural conformation and when denatured. As outlined in this report for one such pre-determined site at the receptor, these conditions were met as follows: The synthetic peptide Tyr-Cys-Glu-Ile-Ile-Val matching in sequence residues 127–132 of the α-subunit of all nicotinic receptors sequenced so far [7] was conjugated to BSA. Mouse monoclonal antibodies and antisera in rabbits were raised against this conjugate. The monoclonal antibodies selected for further studies specifically recognized the peptide portion of the conjugate and the membrane-bound and purified recptor from *Torpedo marmorata* under natural conditions and after denaturation. The obtained rabbit immune sera contained antibodies directed against BSA, the specific peptide–BSA conjugate and the receptor. Response to the latter two antigens remained strong even in the presence of excessive amounts of BSA, indicating that some of the antibodies in the serum recognized antigenic elements that are identical in both the synthetic peptide and the acetylcholine receptor.

Abbreviations: AChR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; mAb, monoclonal antibody

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The related segments of the β,γ and δ-subunits of the receptor from *T. californica* (and by inference of the *T. marmorata* receptor) show partial homology with the hexapeptide of the α-subunit but do not contain a negatively charged residue. Since a carboxylate group is generally assumed to be required as an attachment point for the trimethylammonium moiety of acetylcholine [1,10], the α-peptide therefore contains a central element of distinction from the homologous sequence portions of the other subunits. To test whether the particular peptide region of the α-subunit is part of an acetylcholine binding site, anti-peptide antibody binding to the native receptor was studied in the presence and absence of representative cholinergic ligands. The ligands tested did not interfere with antibody binding. The antibodies directed against the synthetic peptide were also tested as to whether they competed with the binding to the receptor of monoclonal antibodies raised against the whole receptor protein and shown to competitively affect ligand binding [11]. Again, no interference was observed suggesting that residues 127–132 of the α-subunit are not part of the binding sites for cholinergic ligands at the receptor.

2. MATERIALS AND METHODS

Membrane fragments and purified acetylcholine receptor from *T. marmorata*, purification and radioactive labeling of α-cobratoxin and the monoclonal antibodies were performed according to [11–15].

Preparation of peptide-carrier conjugate: The hexapeptide Tyr-Cys-Glu-Ile-Ile-Val was synthesized by a solid phase method [16] and purified by high-performance liquid chromatography. Its purity was further established by amino acid analysis and sequence determination. We thank Dr M. Engelhard and B. Pevec from our institute for the synthesis and characterization of the peptide. The peptide was selectively coupled at its N-terminal to BSA by reaction with glutaraldehyde [17].

Preparation of antisera and monoclonal antibodies: Female New Zealand White rabbits were immunized intradermally with 3 mg peptide–BSA conjugate in Freund's complete adjuvant followed by booster injections (every 3 weeks) of the same amount of antigen in incomplete adjuvant.

To obtain monoclonal antibodies to the synthetic peptide, young female Balb/c mice were injected intravenously with 3 mg peptide–BSA conjugate. Three days after a booster injection, the spleens of the immunized mice were removed and cell hybridization, cell cloning and clone selection were performed as in [11,15].

Antibody binding assays: Enzyme-linked immunosorbent assays (ELISA) of monoclonal antibodies were performed according to [11] except that the commercially available screening kit NEN-NEI 602 (NEN) employing *o*-phenylendiamine as substrate was used. Purified α- and δ-subunits were kindly provided by Dr H.-W. Meyers from our laboratory. ELISAs of rabbit immune sera were performed by the same procedure except that anti-rabbit IgG antibodies (NEN, Dakopatts) were employed.

3. RESULTS AND DISCUSSION

The hexapeptide Tyr-Cys-Glu-Ile-Ile-Val was synthesized and coupled to BSA using glutaraldehyde as cross-linking agent [17]. Antibodies against the conjugate were raised by conventional immunisation of rabbits and by the murine hybridoma technique [18]. Hybridoma clones selected for further studies showed high readings in ELISAs with the peptide-BSA conjugate and the acetylcholine receptor as antigen both before and after heat denaturation, but only background readings with BSA as antigen (table 1). Thus, the selected cell-lines P1–P3 produced antibodies specific for the peptide region of the conjugate, and these antibodies cross-reacted with the native and denatured receptor. The antibodies appeared to exclusively react with the α-subunit of the receptor (fig.1). Since other regions of this subunit have at most two positions in common with the selected hexapeptide, specific recognition by the anti-peptide antibody of the sequence segment homologous with the synthetic peptide is very likely.

The obtained mouse monoclonal antibodies to the hexapeptide were tested as to whether their binding to the receptor can be blocked by rabbit immune sera raised against the same synthetic peptide or against the whole receptor protein. As shown in

Table 1

Binding of monoclonal antibodies to selected antigens[a]

Hybridoma clone		Antigen				
mAb	Internal code	Peptide conjugate	BSA	Acetylcholine receptor		
				Purified	Membranes	Denatured
P1	C1-NII-A1	2.0	0.2	2.6	2.5	2.6
P2	C1-NIII-A1	2.6	0.4	2.6	2.6	2.3
P3	C2-NI-C2	2.6	0.5	2.6	2.6	2.3
P5	C1-NII-B3	2.0	0.2	0.7	1.2	0.9
P6	C1-NI-A1	2.6	2.6	2.6	2.6	2.6
P7	C1-XI-A1	0.4	2.6	0.2	0.7	0.4

[a] Absorbance readings at 490 nm employing the ELISA described in section 2. Readings of 0.1–0.5 A_{490} units were considered background

fig.2, the serum from rabbits immunized with the specific hexapeptide interfered the strongest while only weak interference was observed with an anti-receptor immune serum. Thus, the preselected sequence segment recognized by the anti-peptide antibodies appears to be located at the receptor surface.

To test whether the preselected sequence segment is related to a binding site for cholinergic ligands, binding of monoclonal anti-peptide antibodies to the receptor was studied in the presence and absence of representative cholinergic ligands (α-cobratoxin, carbamoylcholine, decamethonium, tubocurarine). As shown in fig.3, these ligands even at very high concentrations did not compete with binding of the anti-peptide antibodies to the receptor. Thus, the sequence segment homologous with the synthetic peptide apparently

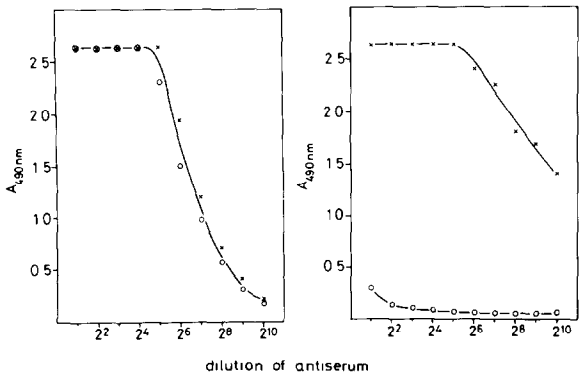

Fig.1. Enzyme-linked immunosorbent assay of anti-peptide antibodies with purified α-subunits (×) and δ-subunits (○) of the AChR from *T. marmorata*. Serial dilution curves of (left) anti-receptor antiserum and (right) anti-hexapeptide antiserum. Specificity for the α-subunit is observed only with the anti-peptide antibodies (right) but not with the anti-receptor serum (left).

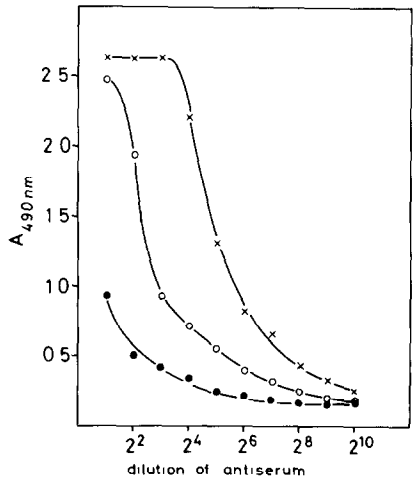

Fig.2. Competition binding to membrane fragments from *T. marmorata* of mAb P3 and rabbit immune sera. Serial dilution curves of mouse hybridoma culture supernatant mAb P3 in the presence of rabbit (●) anti-hexapeptide antiserum (1:20 in PBS), (○) anti-receptor antiserum (1:100 in PBS), and (×) preimmune serum.

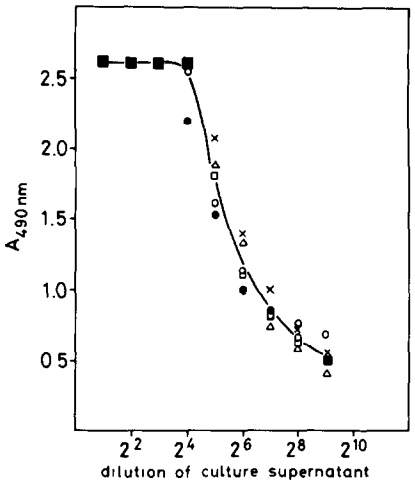

Fig.3. Binding of mAb P3 to AChR-rich membrane fragments in the presence and absence of representative cholinergic ligands. The serial dilutions were obtained by first adding 200 μl of the respective ligand solution to each cuvette of a strip of 10. After preincubation for 15 min (toxin 2 h), 200 μl of culture medium was added to cuvette 1 and mixed; 200 μl of the contents was then transferred to cuvette 2, and so on. (×) Serial dilution in the absence of ligand; (○) α-cobratoxin at initially 1 μM; (△) carbamoylcholine at initially 1 mM; (□) tubocurarine at initially 1 mM; and (●) decamethonium at initially 1 mM.

does not contain structural elements of a binding site for cholinergic ligands.

This conclusion is supported by competition binding studies of a rabbit anti-peptide serum and the same representative ligands to the receptor (not shown). In addition, application of the rabbit serum permits testing whether the sequence segment defined by the synthetic peptide overlaps with any of the known antigenic sites at the receptor [11,19]. Since we did not observe any competition in binding between the rabbit serum and antibodies from our library of monoclonals [11], such overlap apparently does not occur.

4. CONCLUSIONS

We describe here an immunogenic approach to the mapping of functional sites at the surface of the receptor. Since antigenic sites are defined by only a few amino acids [9,22], the employed method of site localization is the more reliable the shorter the synthetic peptides used (with the limitation of a minimal number of 4–6 amino acids). Long peptides, in contrast, may provide several or many antigenic determinants with homologies to other sequence regions of the receptor. This difference in approach to previous studies of similar aim [23] should be emphasised.

Antigenic site analysis in the way described here is limited in local resolution to the minimal size of the peptide required to evoke an immune response. In addition, only surface regions of a protein can be probed in this way. In these respects the method is inferior to site-specific chemical modification and to site-directed mutagenesis. However, the advantage of the immunogenic approach lies in its simplicity and in the additional value specific surface markers to a functional site may have in the quantitative analysis of this function.

Several models of the 3-dimensional structure of the acetylcholine receptor have been proposed [7,24–27]. These are rather specific with respect to the transmembrane regions of the receptor but vague with respect to the structure of the extramembranous regions. Several alternative sequence regions can therefore be envisioned as the positions of the ligand binding sites [10,26,27]. The immunogenic approach described here provides a rapid means to test such preconceived ideas on the structure-function relationship of receptor proteins with known primary structure.

In the specific case of the preselected sequence segment of the acetylcholine receptor (residues 127–132) we conclude from our studies that it does not contain structural elements involved in ligand binding.

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REFERENCES

[1] Maelicke, A. (1984) Angew. Chem. Int. Ed. Engl. 23, 195–221.

[2] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) Nature 299, 793–797.
[3] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, T., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) Nature 301, 251–255.
[4] Claudio, T., Ballivet, M., Patrick, J. and Heinemann, S. (1983) Proc. Natl. Acad. Sci. USA 80, 1111–1115.
[5] Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) Nature 302, 528–532.
[6] Devillers-Thiéry, A., Giraudat, J., Bentaboulet, M. and Changeux, J.-P. (1983) Proc. Natl. Acad. Sci. USA 80, 2067–2071.
[7] Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983) Nature 305, 818–823.
[8] Takai, T., Noda, M., Furutani, Y., Takahashi, H., Notahe, M., Shimizo, S., Kayanu, T., Tanabe, T., Tanaka, K., Hirose, T., Inayama, S. and Numa, S. (1984) Eur. J. Biochem. 143, 109–115.
[9] Atassi, M.Z. (1978) Immunochemistry 15, 909–936.
[10] Smart, L., Meyers, H.-W., Hilgenfeld, R., Saenger, W. and Maelicke, A. (1984) FEBS Lett. 178, 64–68.
[11] Watters, D. and Maelicke, A. (1983) Biochemistry 22, 1811–1819.
[12] Fels, G., Wolff, E. and Maelicke, A. (1982) Eur. J. Biochem. 127, 31–38.
[13] Rüchel, R., Watters, D. and Maelicke, A. (1981) Eur. J. Biochem. 119, 215–223.
[14] Maelicke, A., Fulpius, B.W., Klett, R.P. and Reich, E. (1977) J. Biol. Chem. 252, 4811–4830.
[15] Fels, G., Breer, H. and Maelicke, A. (1983) in: Toxins as Tools in Neurochemistry (Hucho, F. and Ovchinnikov, Y.A. eds) Walter de Gruyter, Berlin.
[16] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[17] Reichlin, M. (1980) Methods Enzymol. 70, 159–165.
[18] Köhler, G. and Milstein, C. (1975) Nature 265, 495–497.
[19] Lindstrom, J. (1983) Neurosci. Commun. 1, 139–156.
[20] Moore, H.-P.H. and Raftery, M.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4509–4513.
[21] Covarrubias, M. and Maelicke, A. (1984) in preparation.
[22] Sutcliff, J.G., Shinnick, T.M., Green, N. and Lerner, R.A. (1983) Science 219, 660–666.
[23] Guillerat, M.A., Barkas, T. and Tzartos, S.J. (1984) FEBS Lett. 168, 143–148.
[24] Finer-Moore, J. and Stroud, R.M. (1984) Proc. Natl. Acad. Sci. USA 81, 155–159.
[25] Guy, H.R. (1984) Biophys. J. 45, 249–261.
[26] Kosower, E.M. (1983) Biochem. Biophys. Res. Commun. 111, 1022–1026.
[27] Kosower, E.M. (1983) FEBS Lett. 157, 144–146.